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STUDIES IN THE PHYSIOLOGY OF THE FUNGI
XI. Bacterial Inhibition by Metabolic Products

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Considerable work has been done on the early phases of growth of bacteria in liquid media. Rahn ('06), Coplans ('07), Penfold ('14), Chesney ('16), Salter ('19), and others have shown quite definitely the factors involved in the lag phase of growth preceding the phase of logarithmic increase. They have demonstrated that the lag can be eliminated if the transfers are made during the period of logarithmic increase, but that certain factors such as difference in temperature, composition of the medium, or the age of the culture will produce a latent period immediately following the transfer.

Data on the later growth periods of bacteria are less extensive. Based on the total number of viable bacteria in the culture, the growth curve can be traced roughly as follows: It rises abruptly at first, which is the phase of logarithmic increase, then ascends more gradually until the peak is reached, and finally descends until the culture is sterile. The influence of inhibitory factors is most clearly seen in the later periods, those following the phase of logarithmic increase, the study of which is of fundamental and practical importance both in killing pathogenic bacteria, that is, hastening the decline in the growth curve, and in prolonging the life of useful cultures, suspending this decline. In the work presented here, emphasis is placed on the later periods of growth and on the influence of the products of a growing culture on the path of the growth curve.

LITERATURE

The literature on the subject of the inhibition of bacteria in culture by their own metabolic products is widely scattered, and the investigational work in this phase of growth studies is very meager. The entire subject is often dismissed with some such statement as "the organisms are finally killed by their own products."

From time to time different investigators have sought to determine if there is a special metabolic product, enzymatic in nature, which inhibits the growth of the organism producing it. One of the earliest publications on this subject appeared by Eijkmann ('04). He grew *Bacillus coli* in gelatin at 37° C., treated it in different ways, and then solidified and reinoculated the gelatin. He concluded that *Bacillus coli* in gelatin produced a diffusible, thermolabile substance which would not pass through a porcelain filter and which inhibited growth of *Bacillus coli* and other organisms, for treatment with ether, subjection to heat, or filtration through a porcelain filter removed some inhibiting substance and permitted a streak growth on the solidified gelatin.

The following year Conradi and Kurpjuweit ('05,'05[a]) extended the work of Eijkmann, finding the same action in bouillon. They called the substance "autotoxin" and applied the theory to the germicidal action found in feces. They reported that the "autotoxin" of *Bacillus coli* was killed by boiling but was virulent up to a dilution of 1:3200 in a 10-hour culture, and that the heated stool filtrate from a paratyphoid patient would support growth of the same *Bacillus paratyphosis* in a 1:50 dilution, but the unheated filtrate only in a 1:400 dilution. Rolly ('06), Passini ('06), and Manteufel ('07) disputed the findings of Eijkmann, also those of Conradi and Kurpjuweit, and held that the existence of inhibitory substances had not yet been proved. Rolly could not repeat the work of Conradi and Kurpjuweit with the same results but found that the filtered half of a 20-hour bouillon culture gave better growth than the cooked half. Manteufel claimed that the loss of necessary food material from the media explained some of the results attributed to "autotoxin." Kruse ('10) summarized these reports and

explained the death of organisms in culture as probably due rather to the exhaustion of the media and the accumulation of well-known metabolic products than to an "autotoxin." He suggested the possibility of the exhaustion of the media and the accumulation of products causing the death of a few of the weaker individuals, which become self-digested, thereby releasing previously formed "autotoxin." Acids and alkalis are reported by him as inhibitory agents, although bouillon in which pneumococci had grown would not support a second growth even on readjusting the reaction.

In connection with some work on the latent period of growth, Chesney ('16) found that pneumococci in plain broth showed marked inhibition 24 hours after inoculation, the number of bacteria decreasing rapidly to zero, but if after 96 hours a portion of the bouillon was filtered through a porcelain filter and reinoculated, no inhibition was evident, indicating that the inhibitory substance was killed or attenuated in 3 days at 37° C.

It is apparent from the literature cited above that results are conflicting concerning the production of an enzymatic "autotoxin," and while the reports favoring the existence of such a product are not conclusive, no other satisfactory explanation for the observed reactions has been demonstrated.

Recent literature has indirectly contributed considerable of value concerning the relationship of acid and alkali to growth and death of bacteria, through the more general use, since 1916, of the hydrogen ion concentration as an expression of acidity of media. Winslow and Lochridge ('06), working on *Bacillus coli* and *Bacillus typhosus*, stated that the toxic effect of inorganic acids, HCl and H_2SO_4, corresponded to their dissociation, but with organic acids, acetic and benzoic, the undissociated molecule was also important, for results did not correspond to the dissociation of the acids. Michaelis ('14) advanced the idea that organisms produce acid to a certain concentration, which he found to be P_H 5.0 with *Bacillus coli* in lactose bouillon, and that they automatically protect themselves against harmful amounts.

Since that time a great deal has been published on final or limiting hydrogen ion concentrations for different organisms,

but only a very little on the effect on growth of changes in hydrogen ion concentration during growth. Clark ('15) determined the final P_H of 16 cultures of *Bacillus coli* in 1 per cent dextrose medium as P_H 4.67–5.16, and Clark and Lubs ('15) in constructing their media for differentiating the members of the colon-aerogenes group showed that a reversion of reaction toward the alkaline may take place, depending on the dextrose, but they did not show the relationship between reversion of reaction and growth. Itano ('16) reported that with *Bacillus subtilis*, *Streptococcus erysipelatus*, and *Streptococcus lacticus* in plain broth, acid was formed in alkaline media and alkali in the acid media, thus bringing the P_H to a certain definite hydrogen ion concentration. Fred and Loomis ('17) showed a wide range of reaction for *Bacillus radicicola*, obtaining good growth between P_H 3.9 and 11.1. They also demonstrated that the hydrogen ion concentration approaches the neutral point during growth. Shohl and Janney ('17) found that P_H 4.6–5.0 was inhibitory for *Bacillus coli* in urine. Ayers, Johnson, and Davis ('18) added streptococci to the list of organisms whose final P_H was demonstrated. They separated the pathogenic from the non-pathogenic forms on the basis of limiting hydrogen ion concentration, the former reaching P_H 5.4–6.0 and the latter P_H 4.6–4.7.

The work of Ayers and Rupp ('18) on simultaneous acid and alkali fermentations showed some interesting P_H curves. They found in a .5 per cent dextrose medium that *Bacillus coli* produced acid to P_H 4.8 but that *Bacillus aerogenes* produced less initial acid and the reaction reverted to P_H 6.5. From quantitative determinations of dextrose and of formic, acetic, lactic, and succinic acids, they explained the reversion of *Bacillus aerogenes* as a fermentation of the organic acids, mostly formic and acetic, to carbonates. With the alkali-forming milk bacteria, they showed alkaline fermentation of citrate, acid fermentation of dextrose, and a practically neutral reaction from the simultaneous fermentation of the citrate and dextrose. Gillespie ('18) found *Actinomyces chromogenus* gave a poor growth at P_H 4.8–5.2 and decreased the hydrogen ion concentration of the media during growth. Wyeth ('18) showed with *Bacillus coli* in glucose bouillon that the final P_H varied with

the initial P_H; i. e., P_H 7.11 progressed to P_H 5.70 and P_H 4.96 went to P_H 4.68. He also showed a difference in critical P_H according to the acid used, whether hydrochloric, acetic, or lactic acid. Wyeth ('19) extended his previous work and found in 2 per cent peptone that an initial range of P_H 4.29–9.37 gave a final range after 216 hours of P_H 5.92–8.55, and that with an initial P_H above 8.48 the production of acid exceeded that of alkali and the reaction approached P_H 8.48. Indole formation was completely inhibited by dextrose, partially by sucrose, and not at all by starch. Avery and Cullen ('19) used the final hydrogen ion concentration to separate strains of *Streptococcus hemolyticus;* 124 human strains attained a final P_H of 4.8–5.3, while 40 dairy strains reached P_H 4.3–4.5.

Considerable work has recently appeared on the pneumococcus. Cullen and Chesney ('18) showed the relation of the growth of pneumococcus in plain broth to hydrogen ion concentration. The bacteria increased to 420,000,000 per cc. in 13.8 hours and then decreased to 160 per cc. in 96 hours. The hydrogen ion concentration increased from P_H 7.70 to P_H 7.03,[1] but these investigators expressed the opinion that the increase in hydrogen ion concentration is not the sole cause of the cessation of growth. Avery and Cullen ('19[a]) showed some interesting reactions of pneumococcus to carbohydrates. One per cent of maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin produced a final P_H of about 5.0. With .4 per cent dextrose, as high an hydrogen ion concentration was attained in 48 hours as with 1 or 2 per cent dextrose. Pneumococcus differed from *Bacillus coli* in that it produced acid in plain broth, and growth ceased at about P_H 7.0. When this culture was readjusted to P_H 7.8 and reinoculated, no growth occurred unless carbohydrate was added, yet the filtrate from a dextrose culture at P_H 5.2 if readjusted to P_H 5.8, 7.0, or 8.0 would again return to P_H 5.2. Growth could only be initiated within certain limits, in carbohydrate media P_H 8.3–6.8 and in plain broth P_H 8.1–7.0. They concluded that the exhaustion of fermentable carbohydrate is only one of the many

[1] To avoid confusion, attention is called to the distinction between the concentration of the hydrogen ions and the symbolic P_H. A numerical increase in hydrogen ions is expressed as a decrease in terms of P_H.

factors involved in the complex phenomenon of growth inhibition.

Lord and Nye ('19) have demonstrated the relation of time to inhibitory action of hydrogen ion concentration with pneumococcus. They found that Pneumococcus Type I was killed in 1 hour at P_H 4.5–4.7, in 3 hours at P_H 5.3, and in 6 hours at P_H 6.15, but survived 6 hours at P_H 6.35, and that between P_H 6.8 and P_H 5.1 there was a direct relation between the P_H and the time required for the death of the pneumococcus. In mixtures of equal quantities of emulsions of washed pneumococci and buffer solutions of different hydrogen ion concentrations they observed very little dissolution of the bacterial cells between P_H 8.0 and P_H 7.0 or between P_H 5.0 and P_H 4.0, but noticed almost complete dissolution in the zone of P_H 6.5– 5.5.

Bunker ('19) published the results of investigations of *Bacillus diphtheriae* extending over several years. The hydrogen ion curves in sugar-free and in 1 per cent dextrose media agree very closely with those of *Bacillus coli* in the experimental work of this report. He also showed that toxin was only produced within a rather narrow hydrogen ion range, P_H 7.8–8.25. The best growth, measured by pellicle formation, was obtained when the initial reaction was P_H 7.3–7.5. Cohen and Clark ('19) investigated the effect of hydrogen ion concentration on the rate of growth of different organisms during the early part of the growth curve, the period of logarithmic increase. Cultures were inoculated into media adjusted over a wide range of varying initial hydrogen ion concentrations, and observed for the first 10 hours of growth. In general, the different organisms reacted similarly. The most marked effect of the hydrogen ion concentration on early growth was found near the critical acid and alkali zones. They reported that with *Bacillus coli* fermentative activity was checked in 1 per cent dextrose bouillon at P_H 5.0, but that growth in plain bouillon was checked at P_H 5.7. They noted evidence of inhibition which obscured their results, but they did not study the inhibitory factors; however, it was found that the period of lag was more pronounced in alkaline than in acid media.

Recent contributions from Besson, Ranque, and Senez ('19), while they do not involve hydrogen ion concentration, advance

some new ideas on sugar relations and fermentation. They worked with *Bacillus coli* in bouillon containing varying amounts of dextrose. With less than .4 per cent dextrose the sugar was all removed in 24 hours and the cultures were viable after 10 days, while with .4 per cent or over the cultures were sterile in 6 days. They reported that fermentation with gas commenced at the time multiplication of the organisms ceased, that acid production started at the same time, and that more than one-half the total acid is produced in 1 hour.

From the literature reviewed it would appear that a correlation of growth curves and P_H curves, with frequent observations during growth, rather than a study of final hydrogen ion concentration, would add to our knowledge of metabolic changes in hydrogen ion concentration and of inhibition during growth.

Technique

The experimental work was planned on the basis of a correlation of the growth of the bacteria with the changes in the hydrogen ion concentration of the media produced during growth. The technique was uniform throughout to make all results comparable. Cultures were grown in Florence flasks of 500, 1000, and 2000 cc. capacity, filled to one-half their capacities for the initial volume of media to insure a uniform and maximum surface. The basic bouillon for all the cultures, designated plain bouillon through the text, consisted of 2.5 per cent bacto-beef and 1 per cent bacto-peptone made up with distilled water according to the Digestive Ferments Company circular of December, 1916. This plain bouillon forms the basis for the different dextrose media, with a few exceptions which are noted in the data.

A culture of *Bacillus coli*, culture FG, kindly furnished from the Dairy Division, United States Department of Agriculture, was used throughout the experimental work with one exception, in which *Bacillus aerogenes*, culture VE from the same laboratory, was substituted.

To avoid the lag phase, transfers from stock agar were grown through two successive cultures of plain bouillon, and the inoculation was made from the second culture between 6 and 10 hours, during its period of logarithmic increase. A uniform tempera-

TABLE I

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN PLAIN AND 1 PER CENT DEXTROSE BOUILLON AT 30° C.

Hours	1 Plain bouillon		2 Plain bouillon and 1% dextrose	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	54,000	7.1	55,000	7.1
12	175,000,000	6.8	268,000,000	5.3
20			281,000,000	5.1
24	320,000,000	7.2	214,000,000	4.9
36	538,000,000	7.5	220,000,000	4.8
48	609,000,000	7.6	189,000,000	4.8
72	450,000,000	7.7	119,000,000	4.8
96	459,000,000	7.8	14,500,000	4.9
120	293,000,000	7.9	11,100	4.9
144	250,000,000	8.1	0	4.9
168	151,000,000	8.1		
192	156,000,000	8.3		
234	125,000,000	8.2		
276	115,000,000	8.3		
348	89,000,000	8.3		
492	69,000,000	8.3		
612	71,000,000	8.3		
1284	53,000,000	8.5		
1800	7,500,000	8.7		

ture of 30° C. was maintained for all cultures throughout the work.

The changes in growth and hydrogen ion concentration in each culture were followed by removing, under aseptic conditions, a 3-cc. sample at 12- or 24-hour intervals, after the flasks had been rotated briskly 30 times to mix the contents thoroughly. One cc. was diluted and plated in triplicate in agar composed of plain bouillon to which 1 per cent dextrose and 1.5 per cent granular agar had been added. The hydrogen ion concentration was determined from the remaining 2 cc. according to the colorimetric method of Clark and Lubs ('17), using the micro-colorimeter described by Duggar ('19). The hydrogen ion concentration is expressed in P_H, or the reciprocal values of Sörensen now in general biological use. Plates were counted after an incubation of 72 hours at 30° C.

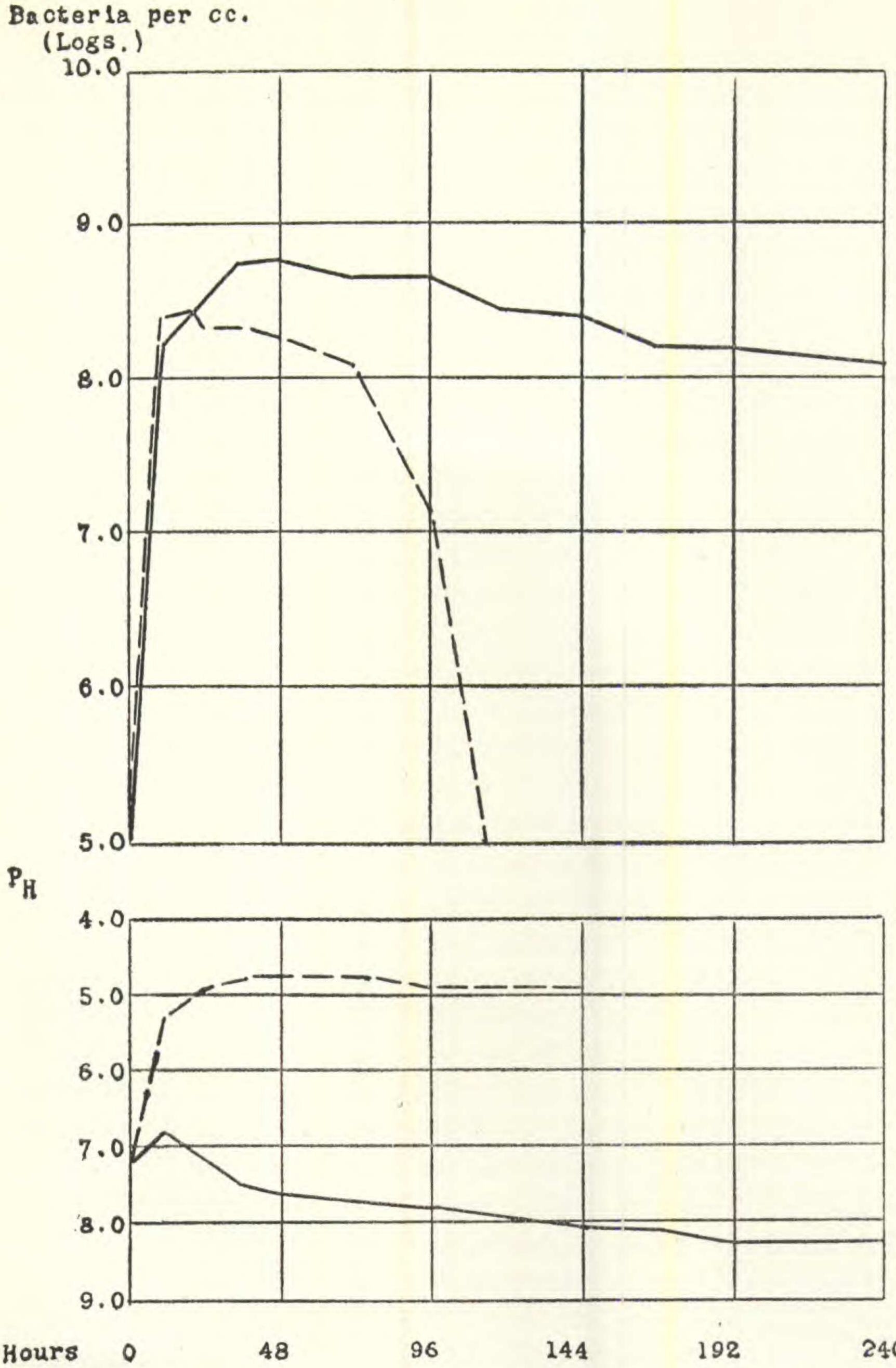

Fig. 1. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C.
———— plain bouillon.
— — — — 1 per cent dextrose bouillon.

EXPERIMENTAL DATA

As a starting point for the experimental work and as a basis for comparison of inhibitory action, one culture in plain bouillon and one culture in this bouillon with 1 per cent dextrose added were inoculated with equal numbers of *Bacillus coli* from the same culture. The resulting growth (expressed in numbers of bacteria per cc.), and the hydrogen ion concentration of the media (expressed in P_H) are recorded in table I. The comparison is more strikingly shown in fig. 1, in which the growth curves are plotted from the logarithms of numbers of bacteria per cc. as given in table I. A comparison of the hydrogen ion curves shows a rapid production of acid from dextrose, attaining P_H 4.8 in 36 hours, but a slower production of alkali in the plain bouillon with the exception of the short acid break at the beginning of the curve. Growth in the dextrose bouillon is more rapid in 12 hours than in the plain bouillon but the maximum is reached in 20 hours, 281,000,000 bacteria per cc. when the P_H is 5.1, and the decline is then very abrupt, terminating in sterility of the culture in 144 hours. In the plain bouillon, the maximum is reached in 48 hours, 609,000,000 bacteria per cc., with a P_H of 7.6. However, after 75 days, although a P_H of 8.7 is attained, there are still 7,500,000 viable bacteria per cc. in the culture. Apparently, then, the more intense inhibition is found in the dextrose rather than in the plain bouillon.

If a bacterial "autotoxin," or any inhibitory action such as Chesney found with pneumococcus in plain broth, is produced by *Bacillus coli*, it would seem, from the results given in table I, to be associated with the dextrose bouillon and not with the plain bouillon. A series of cultures in a 1 per cent dextrose medium were observed for the purpose of determining any variation in inhibitory action during growth and death. The results are given in table II and illustrated in fig. 2. Flask 1, the parent culture, contained the same 1 per cent dextrose bouillon as Culture 2 of table I. Subcultures of 200 cc. each were removed from the parent cultures at the times indicated, commencing before the point of maximum growth was reached and covering a range well into the period of rapid death. The reaction of the subcultures was readjusted to approximately neutral with sterile N/1 NaOH to eliminate the acidity factor,

TABLE II

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN 1 PER CENT DEXTROSE BOUILLON AT 30° C., SUBCULTURED AT INTERVALS

Hours	Parent culture		Subcultures									
			1		2		3		4		5	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	55,000	7.1										
12	268,000,000	5.3										
14			268,000,000	7.6								
20	281,000,000	5.1			281,000,000	7.1						
24	214,000,000	4.9	511,000,000	5.0	295,000,000	5.4						
26							214,000,000	7.5				
36	220,000,000	4.8	557,000,000	4.9	428,000,000	4.9	339,000,000	5.1				
48	189,000,000	4.8	488,000,000	4.9	396,000,000	4.8	342,000,000	4.8				
72	119,000,000	4.8	94,000,000	4.8	174,000,000	4.8	136,000,000	4.8	123,000,000	7.3		
84									261,000,000	6.7		
96	14,500,000	4.9	8,900,000	4.9	5,500,000	4.9	23,000,000	5.0	264,000,000	5.3	16,900,000	7.3
108											274,000,000	5.7
120	11,100	4.9	1,230,000	4.9	610,000	4.9	1,980,000	4.9	194,000,000	4.9	227,000,000	5.1
144	0	4.9	38,300	4.9	29,600	4.9	330,000	4.9	148,000,000	4.9	134,000,000	4.9
168			9,400	4.9	1,850	4.9	40,000	5.1	84,000,000	4.9	114,000,000	4.9
192			333	4.9	39	4.9	2,900	4.9	62,000,000	4.9	89,000,000	4.9
216			21	4.9	3	4.9	408	4.9	19,000,000	4.9	38,000,000	4.9
240			1	4.9	0	4.9	48	4.9	2,760,000	4.9		
264									350,000	5.1	70,000	4.9
288							0	4.9	14,000	5.0		
312									552	5.0	26,400	4.9
336									84	4.9		
360									9	4.9	310	4.9
408									0	5.0	0	4.9

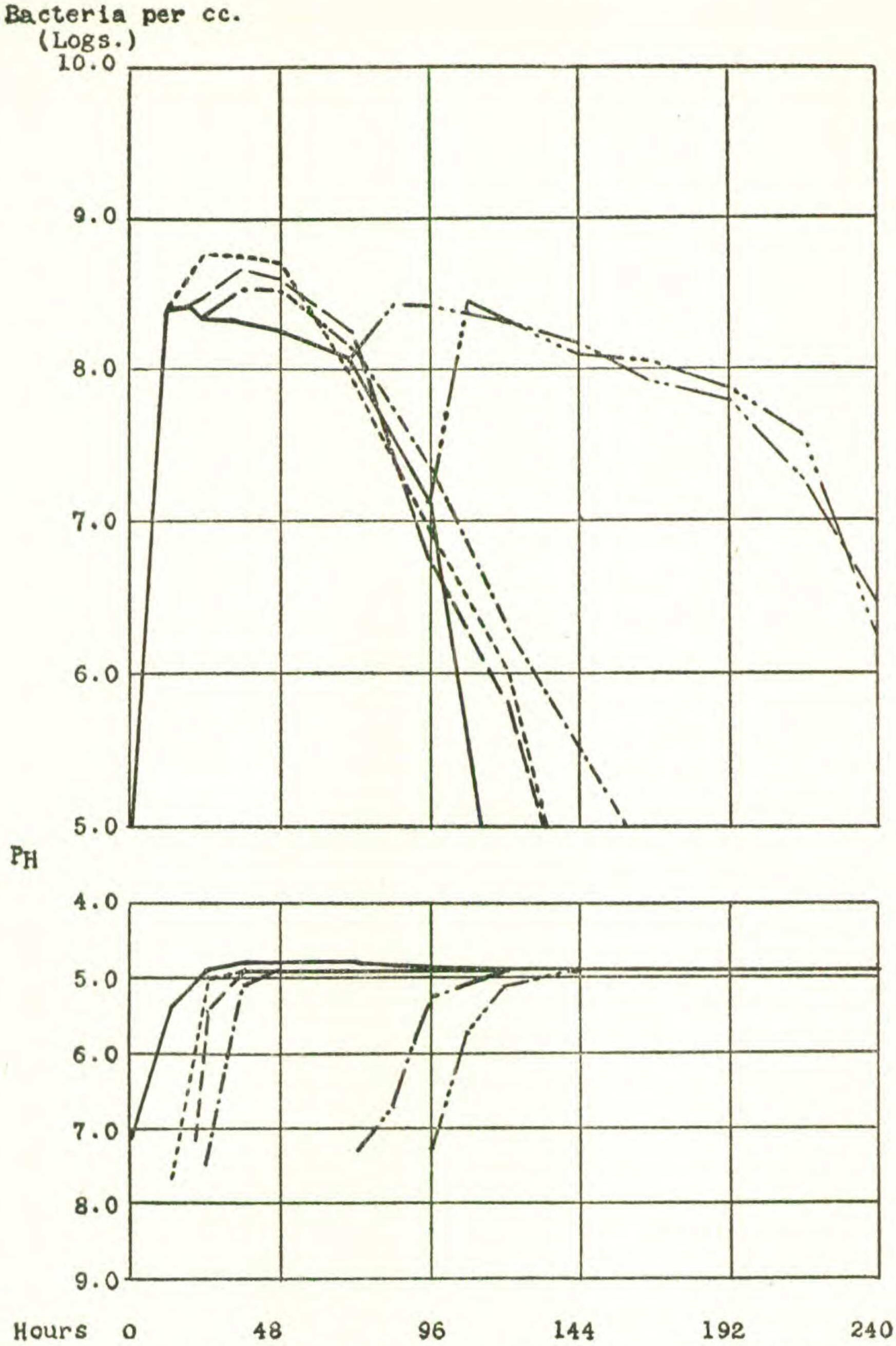

Fig. 2. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., 1 per cent dextrose bouillon, subcultured at intervals.

———— parent culture.
- - - - - - - - Subculture 1.
— — — — Subculture 2.
—·—·—· Subculture 3.
—··—··— Subculture 4.
—···—··· Subculture 5.

and the subcultures were then observed as new cultures. The points of maximum growth for the different subcultures in order are 557,000,000, 428,000,000, 342,000,000, 264,000,000, and 274,000,000 bacteria per cc., so that the subcultures fall in a regular series of decreasing maximum growths, with the exception of Culture 4 whose maximum might have occurred unobserved between 84 and 96 hours. There is nothing distinctive in the changes in hydrogen ion concentration, each subculture producing acid to P_H 4.9 in 24 to 36 hours. It would seem, then, from this series of subcultures that some factor besides hydrogen ion concentration caused an inhibition of the growth, increasing with the age of the culture.

To determine the influence of the exhaustion of the medium as a factor in the inhibitory action shown in fig. 2, another similar series was observed. Four subcultures of 200 cc. each were removed from a parent culture at 96 hours and treated in different ways. All were readjusted to approximately neutral with N/1 NaOH. In addition 50 cc. of plain bouillon condensed 5 times was added to No. 2 (200 cc.), making a total volume of 250 cc. No. 3 received 50 cc. of the condensed bouillon and 1 per cent dextrose. No. 4 received the same nutrients as No. 3 and was then sterilized for 15 minutes at 120° C. to kill any "autotoxin" or inhibitory enzymatic substance, and reinoculated as a new culture. The results are contained in table III and fig. 3. The changes in hydrogen ion concentration are very uniform, falling on almost the same line, an increase until P_H 4.9 is reached in 48 hours. The exhaustion of the medium is shown, however, by the increased growth both in the culture with the bouillon replenished and in the replenished bouillon with dextrose added. The addition of dextrose shows almost no advantage over the addition of concentrated bouillon alone, so that dextrose is not considered an important factor at this time. Acid production from P_H 7.3 to 4.9 in Subcultures 1 and 2 where the dextrose was not replenished shows that all the dextrose had not been fermented in the parent culture at 96 hours. The maximum in Subculture 3 (table III) of 545,000,000 bacteria per cc. compares very favorably with 557,000,000 in Subculture 1 of table II, so that it would appear that the exhaustion of the nutrients contained in the plain bouillon was a very im-

TABLE III

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI IN 1 PER CENT DEXTROSE BOUILLON AT 30° C., SUBCULTURES WITH ADDED NUTRIENTS

Hours	Parent culture		Subcultures							
			1		2		3		4	
					Concentrated plain bouillon		Concentrated plain bouillon and 1% dextrose		Concentrated plain bouillon and 1% dextrose (sterilized and reinoculated)	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	82,000	6.8								
12	288,000,000	5.3								
24	250,000,000	4.8								
48	271,000,000	4.7								
72	87,000,000	4.7								
96	16,900,000	4.7	16,900,000	7.3	18,000,000	7.3	13,500,000	7.2	360,000	6.9
108			274,000,000	5.7	522,000,000	5.5	545,000,000	5.5	309,000,000	5.5
120	21,400	4.7	227,000,000	5.1	527,000,000	5.1	526,000,000	5.1	298,000,000	5.1
144	4,000	4.7	134,000,000	4.9	432,000,000	4.9	496,000,000	4.9	225,000,000	4.9
168			114,000,000	4.9	166,000,000	4.9	160,000,000	4.9	161,000,000	4.9
192			89,000,000	4.9	102,000,000	4.9	128,000,000	4.9	105,000,000	4.9
216			38,000,000	4.9	5,000,000	4.9	8,000,000	4.9	31,000,000	4.9
264			70,000	4.9	150,000	4.9	162,000	4.9	10,000	4.9
312			26,400	4.9	1,500	4.9	310	4.9	0	4.9
360			310	4.9	0	4.9	0	4.9		
408			0	4.9						

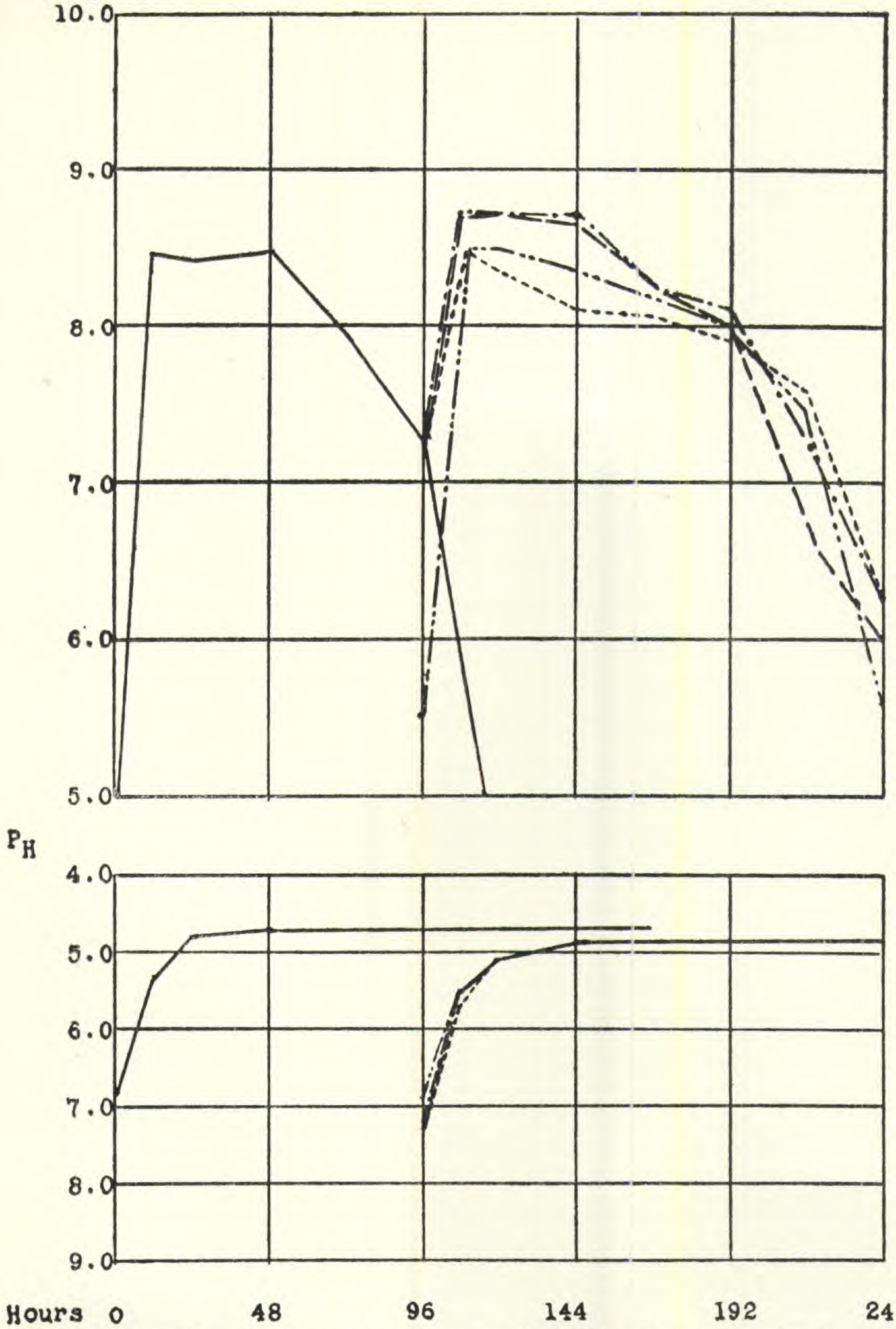

Fig. 3. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., 1 per cent dextrose bouillon, subcultures with added nutrients.

———— parent culture.
- - - - - - - - Subculture 1.
— — — — Subculture 2.
—·—·—· Subculture 3.
—· ·—· ·— Subculture 4.

portant factor in causing the increasing inhibitory action up to 96 hours. Subculture 4, with the same added nutrients as Subculture 3 but sterilized and reinoculated, did not attain the growth of Subculture 3, probably because of the small inoculation. However, the fact that the sterilized subculture did not surpass the unsterilized would indicate that in the parent culture or in the other subcultures the inhibition was not due to a substance which could be killed by sterilizing.

Some investigators have reported that the inhibitory action disappeared on standing and that a good growth was attained upon reinoculation, although the acidity was unaltered. To check this with *Bacillus coli*, 3 to 5 days after the cultures reported in table III became sterile, Subcultures 1, 3, and 4 were mixed together and divided into three equal 200-cc. portions, designated Cultures A, B, and C. Culture A was unchanged; Culture B was sterilized 15 minutes at 120° C.; and Culture C was readjusted to P_H 7.3. All were inoculated from the same culture of *Bacillus coli* with approximately 275,000 bacteria per cc. The growth and hydrogen ion concentration changes are recorded in table IV and fig. 4. Where unaltered, the hydrogen ion concentration in Cultures A and B is P_H 5.1 at inoculation, progressing to P_H 4.9 in a short time. Death of the bacteria occurs shortly, with very little difference between the sterilized and unsterilized cultures. In Culture C, unsterilized but with acidity corrected to P_H 7.3, growth and formation of acid occur similar to that in a new culture. Normal growth when the acidity was adjusted to neutral and no growth when it was not, both in the sterilized and unsterilized cultures, would indicate that no thermolabile substance which disappears on standing was present and that the hydrogen ion concentration of the medium was the important inhibitory factor.

The combined results expressed in the four tables might be summarized as follows: Inhibition to the point of death occurred only in dextrose bouillon in conjunction with acid formation, and not in plain bouillon with alkali formation. A slight inhibitory action was found in dextrose bouillon, increasing with the age of the culture up to 96 hours, which was not attributable to acid but which probably was due to a diminution of the nutrients in the medium. No indication was found of an in-

TABLE IV

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., DEXTROSE BOUILLON, REINOCULATED

Culture	A		B		C	
Treatment	None		Sterilized		Acidity adjusted	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	275,000	5.1	286,000	5.1	261,000	7.3
12	11,400	5.0	15,200	4.9	280,000,000	5.5
24	7,200	5.0	3,800	4.9	320,000,000	5.5
36	3,600	4.9	650	4.9	340,000,000	5.3
48	530	4.9	75	4.9		
60	30	4.9	2	4.9	264,000,000	5.1
84	0	4.9	0	4.9	104,000,000	5.1
108					89,000,000	5.1
132					57,000,000	5.1
156					9,500,000	5.1
180					660,000	5.1
204					36,600	5.1
228					5,400	5.1
252					280	5.1
300					0	5.1

hibitory substance which was destroyed by sterilization or inactivated on standing. The evidence of these results is against an "autotoxin" theory and points toward the hydrogen ion concentration as the predominating inhibitory factor in the experiments cited.

The balance of the experimental work concerns the relation of hydrogen ion concentration to inhibition. To counteract the influence of acid and alkali produced during growth, and thus to study their action by comparison, two cultures were observed in which the acid or alkali formed was neutralized at frequent intervals. The media used was the same as that reported in table 1, one culture of plain bouillon and the other of 1 per cent dextrose bouillon, 500 cc. each in 1000-cc. flasks. The acid produced in the dextrose culture was neutralized at 12-hour intervals by the addition of N/1 NaOH and the hy-

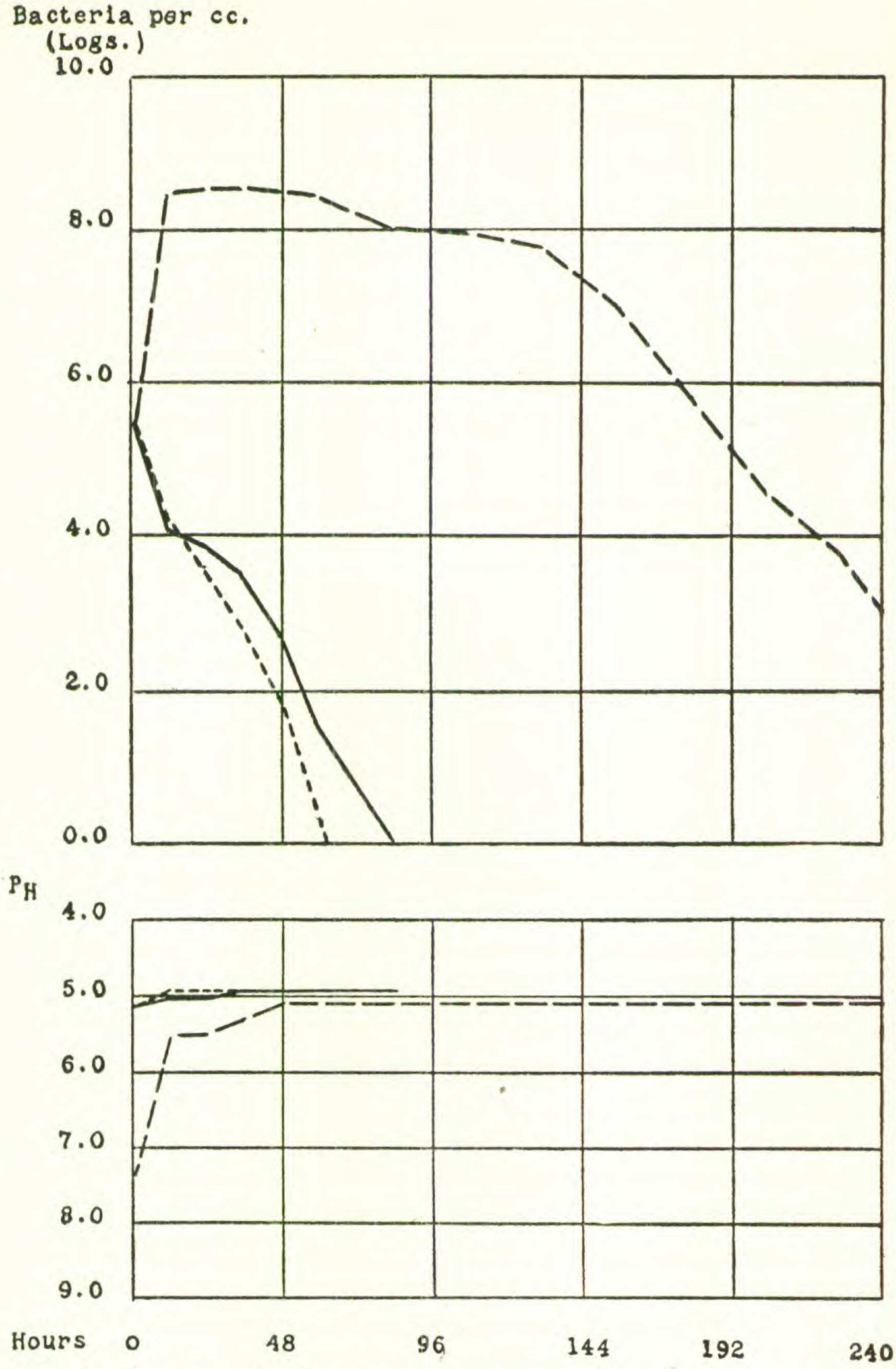

Fig. 4. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., dextrose bouillon, reinoculated.

———— Culture A, untreated.
- - - - - - - - Culture B, sterilized.
— — — — Culture C, acidity adjusted.

TABLE V

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., PLAIN AND 1 PER CENT DEXTROSE BOUILLON, NEUTRALIZED AT INTERVALS

	1 Plain bouillon			2 1% Dextrose bouillon		
Hours	Bacteria per cc.	Cc. of N/5 HCl added	P_H	Bacteria per cc.	Cc. of N/1 NaOH and N/5 HCl added	P_H
					N/1 NaOH	
0	57,000		7.1	54,000		7.1
12	184,000,000		7.0	259,000,000		5.3
14					4.0	6.5
24	391,000,000		7.3	347,000,000		5.1
26		3	6.9		6.3	7.1
36	552,000,000		7.4	507,000,000		5.1
38		5	7.0		6.5	6.9
48	660,000,000		7.3	612,000,000		5.1
50		5	6.7		7.0	7.0
60			7.1			5.3
62		3	6.7		7.0	6.7
72	631,000,000		7.1	692,000,000		5.7
74		3	6.7		7.0	7.3
96	684,000,000		7.2	728,000,000		7.5
					N/5 HCl	
98		3	6.8		3.0	7.3
120	696,000,000		7.3	532,000,000		7.3
122		3	6.7		3.0	7.3
144	680,000,000		7.3	455,000,000		7.5
146		3	6.9		16.0	6.9
168	570,000,000		7.2	487,000,000		7.5
192	693,000,000		7.1	618,000,000		7.3
234	437,000,000		7.3	885,000,000		7.5
236		3	6.9		15.0	7.0
276	450,000,000		7.1	794,000,000		7.6
278		4	6.8		20.0	7.1
348	327,000,000		7.1	608,000,000		7.7
350		3	6.5		25.0	6.7
492	256,000,000		7.1	441,000,000		8.1

drogen ion concentration was determined before and after each addition. The plain bouillon was treated similarly, correcting the alkali with N/5 HCl. The growth in bacteria per cc., the hydrogen ion concentration and the cc. of acid or alkali added

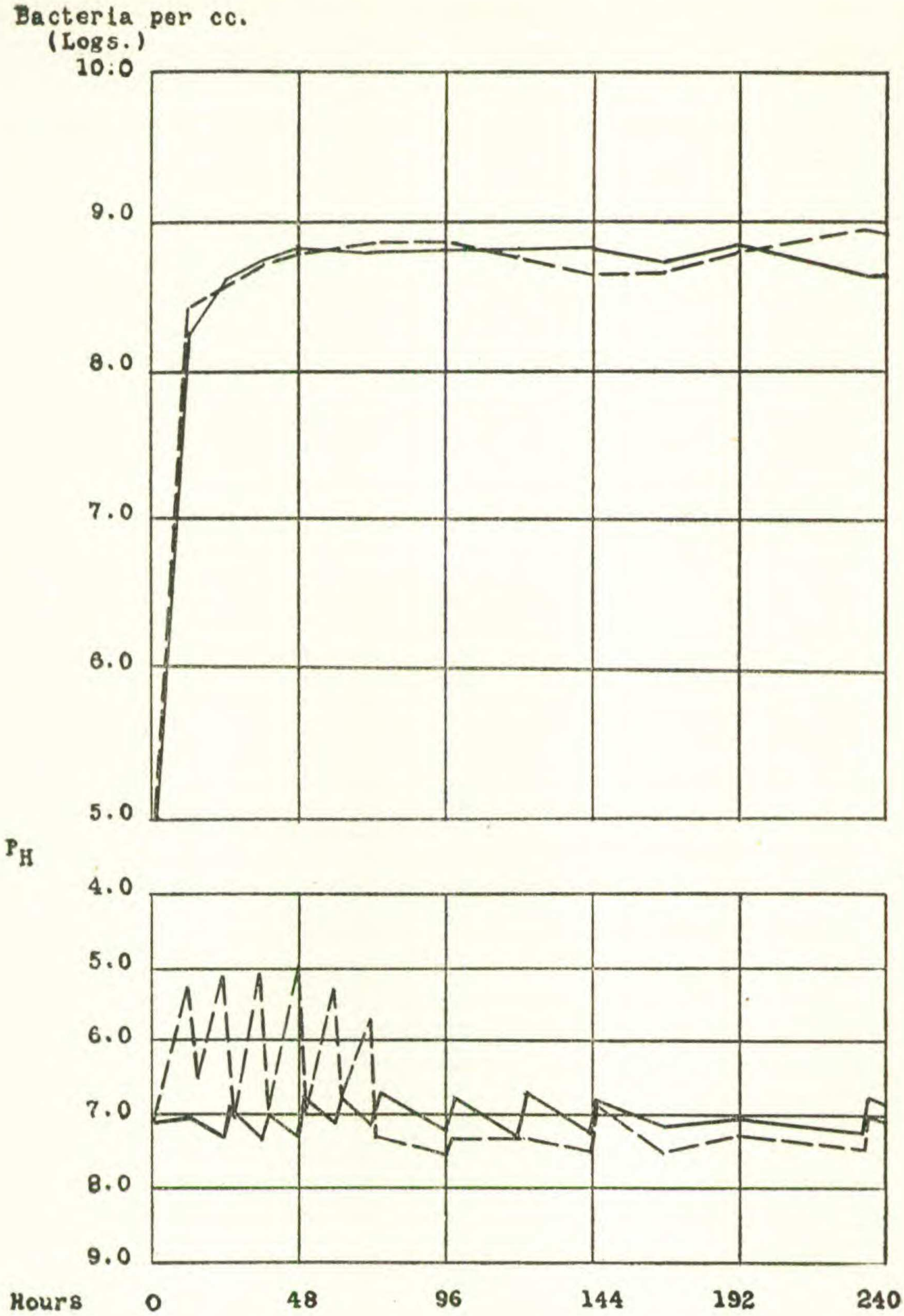

Fig. 5. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., plain and 1 per cent dextrose bouillon, neutralized at intervals.

———— plain bouillon.

— — — — 1 per cent dextrose bouillon.

are given in table v. A more striking representation of the changes in the concentration of the hydrogen ions is shown in the curves in fig. 5. Growth in both cultures is practically parallel, with the dextrose culture reaching the highest point—885,000,000 per cc. at 234 hours. From a comparison of these growth curves with those of fig. 1, it is quite evident that neutralizing the acid or alkali prolongs the growth at a higher level.

The P_H curve for the dextrose culture shows an abundant production of acid, going as high as P_H 5.1 several times. Between 72 and 96 hours, however, the formation changed to alkali, and N/5 HCl was added to neutralize. Table v shows that 37.8 cc. of N/1 NaOH were required to neutralize the acid from 1 per cent dextrose and that in the same time, 96 hours, 19 cc. of N/5 HC1 were used in neutralizing the alkali in the plain bouillon, giving a ratio of 189 to 19, or approximately 10 to 1. Theoretically, then, one-tenth of the dextrose, or .1 per cent dextrose, would furnish just enough acid in 96 hours to neutralize the alkali formed in plain bouillon, and would hold at neutral the hydrogen ion concentration of a growing culture which was fermenting dextrose, if the dextrose were added in small amounts at frequent intervals.

On this basis a culture was started in plain bouillon. The amounts of dextrose added, the growth, and the P_H values are given in table vi and illustrated in fig. 6. By 72 hours the hydrogen ion concentration had demonstrated that the theoretical amount, .025 per cent of dextrose every 24 hours, did not furnish sufficient acid to neutralize the alkali, so the amount of dextrose was increased and the intervals between additions shortened to meet the needs of the culture. The reaction, with each addition of sugar, depends on the acid fermentation of the sugar and a subsequent alkali formation, as illustrated by the P_H curves between 48 and 72 hours and between 96 and 108 hours. This alkali formation was reversed by the addition of more sugar at the proper time. Although the theoretical calculation was upset by the increased growth, the P_H curve demonstrates that it was possible to hold the hydrogen ion concentration within a very narrow zone around the neutral point. The growth was very rapid, reaching 1,550,000,000 bacteria per cc. at 48 hours, or 2½ times as many bacteria as Culture 2,

TABLE VI

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., DEXTROSE ADDED AT INTERVALS

Hours	Bacteria per cc.	P_H	Cc. of 12.5% dextrose added	% Dextrose in 500 cc. of medium	Hours	Bacteria per cc.	P_H	Cc. of 12.5% dextrose added	% Dextrose in 500 cc. of medium
0	309,000	7.1	1.0	.025	252			1.2	.03
12	363,000,000	6.4			264	2,850,000,000	7.0	2.4	.06
24	923,000,000	7.0	1.0	.025	288		6.7	2.2	.055
36	1,360,000,000	6.9			312	1,700,000,000	6.7	2.0	.05
48	1,550,000,000	7.1	1.0	.025	336		6.6	1.0	.025
60	1,400,000,000	7.0			360		6.7		
72	1,512,000,000	7.3	1.0	.025	384	2,014,000,000	7.0	1.0	.025
84	1,230,000,000	7.3	1.5	.0375	408		7.0	1.0	.025
96	1,550,000,000	7.3	2.0	.05	432	1,840,000,000	7.1	1.0	.025
104		6.7			480		7.3	1.5	.0375
108		7.0	1.0	.025	504	1,705,000,000	7.2	1.8	.045
120	2,250,000,000	7.3	2.0	.05	532		7.2	1.5	.0375
132		6.8	1.0	.025	552	2,650,000,000	7.1	1.6	.04
144	3,125,000,000	7.0	1.5	.0375	564		6.5		
156	3,200,000,000	6.7	1.0	.025	576		7.0	1.6	.04
168	3,750,000,000	7.0	1.3	.0325	600	1,675,000,000	7.1	2.0	.05
180		6.9	1.2	.03	648		7.3	2.0	.05
192	3,250,000,000	6.9	1.2	.03	672	2,100,000,000	7.0	2.0	.05
204		6.9	1.1	.0275	720		7.2	2.0	.05
216	3,425,000,000	7.0	1.2	.03	744		6.7	1.0	.025
228		7.0	1.2	.03	768	1,500,000,000	6.7	1.0	.025
240	2,650,000,000	7.0	1.2	.03	816		7.1	1.3	.0325
					840	2,300,000,000	7.0	1.2	.03

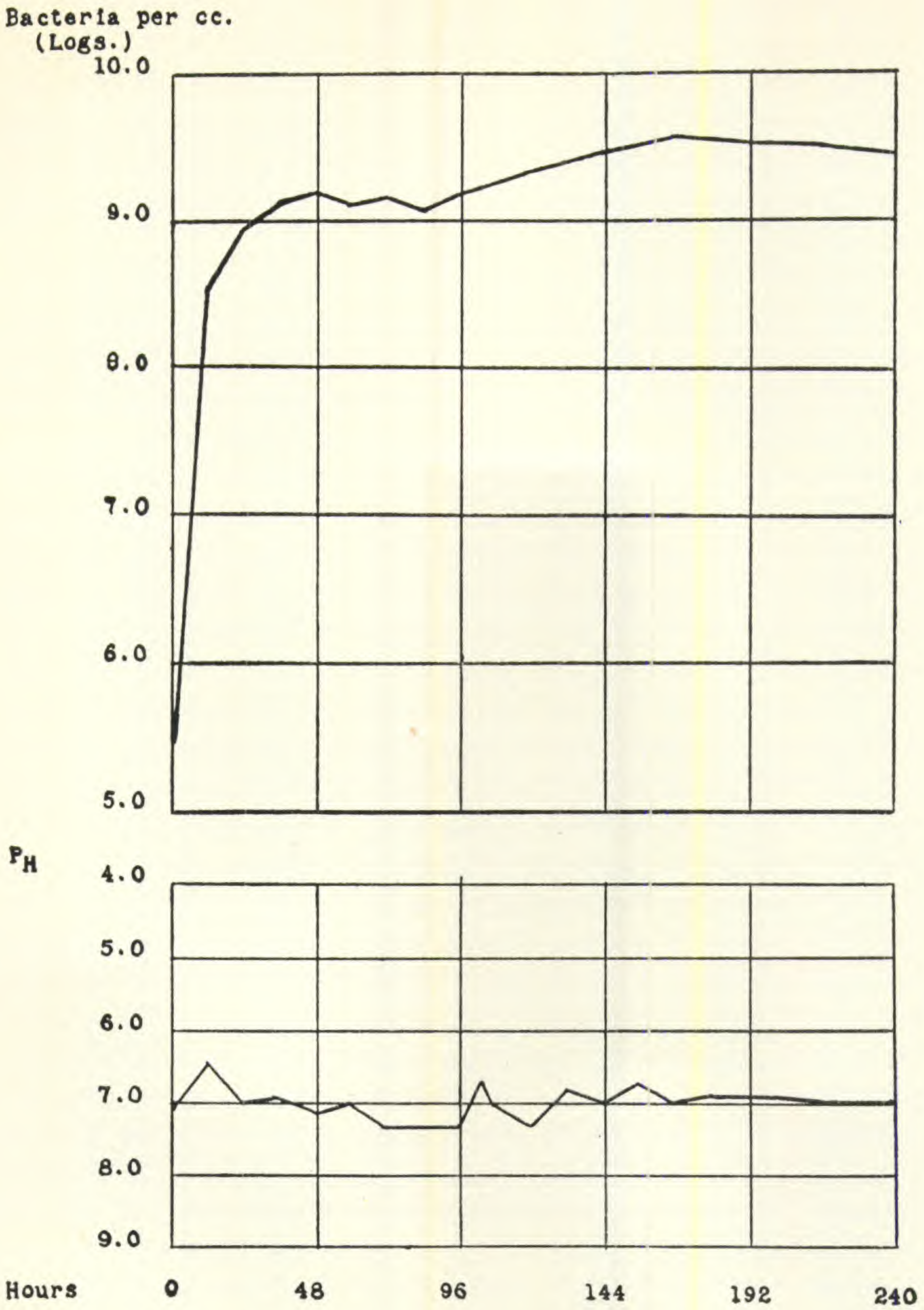

Fig. 6. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., dextrose added at intervals.

table v, produced in the same period of growth. Probably this increased growth explains the more rapid utilization of the dextrose than was calculated. The maximum growth was attained at 168 hours—3,750,000,000 bacteria per cc. Thus this culture showed the least inhibition of any of the experimental cultures and serves as a standard for comparison with the others.

To study more in detail the effect of small amounts of acid, a series of cultures was observed in which the only individual variation was in the initial amount of dextrose. To 250 cc. of plain bouillon in each of five 500-cc. flasks were added respectively .05, .1, .15, .2, and .3 per cent of dextrose, and all were inoculated from the same culture tube of *Bacillus coli*. The growth and changes in P_H are presented in table VII. The cultures are numbered, as indicated in the table, from 1 to 5 in order of increasing amounts of dextrose. Cultures 2, 4, and 5 are plotted in fig. 7 as representative of the series. The P_H curves show that acid was produced in each culture and that the amount of acid formed corresponded to the amount of dextrose provided. The cultures formed a regular series of increasing acidities. Following the acid production there was a reversion of the reaction toward alkalinity which was quite rapid in the first four cultures but slower in Culture 5, where a P_H of 5.1 was maintained from 24 to 96 hours. Comparing the growth curves of the five cultures during the period from inoculation to 48 hours, it is seen that Culture 2 makes the best growth and that Cultures 3, 4, and 5 follow in order. It would appear, then, that .1 per cent of dextrose or less is stimulative in effect and that there is no acid injury from a short exposure to P_H 5.9 (Culture 2). There is, however, some acid inhibition from a P_H of 5.5 (Cultures 3 and 4) and quite a marked inhibition—sufficient to cause some decrease in numbers—from 3 days' exposure to P_H 5.1 (Culture 5). In each case the growth curve ascended as the P_H curve descended toward the alkaline side. The maximum growth was approximately the same for all the cultures—1,400,000,000 to 1,800,000,000 bacteria per cc.—and was reached when the hydrogen ion concentration fell in a zone between P_H 7.0 and 7.6. At the point of maximum growth the hydrogen ion concentrations for the cultures in order were

TABLE VII

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., VARIATION IN INITIAL DEXTROSE

Cultures	1		2		3		4		5	
Dextrose	.05%		.10%		.15%		.20%		.30%	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	300,000	7.1	340,000	7.1	325,000	7.1	335,000	7.1	270,000	7.1
12	392,000,000	6.4	399,000,000	5.9	321,000,000	5.5	329,000,000	5.5	333,000,000	5.4
24	992,000,000	6.9	1,060,000,000	6.7	881,000,000	6.1	593,000,000	5.5	529,000,000	5.1
36	1,392,000,000	7.3	1,768,000,000	7.1	1,200,000,000	6.8	1,132,000,000	6.0	624,000,000	5.1
48	1,376,000,000	7.5	1,330,000,000	7.4	1,292,000,000	7.3	1,496,000,000	7.1	648,000,000	5.1
72	1,360,000,000	7.6	1,360,000,000	7.6	1,680,000,000	7.6	1,824,000,000	7.5	512,000,000	5.1
96	853,000,000	8.1	960,000,000	8.2	1,270,000,000	8.2	1,350,000,000	8.2	454,000,000	5.1
120	516,000,000	8.4	520,000,000	8.5	672,000,000	8.5	472,000,000	8.4	516,000,000	5.2
144										5.5
168	295,000,000	8.5	275,000,000	8.5	243,000,000	8.5	248,000,000	8.5	635,000,000	5.7
192										5.9
216									1,055,000,000	6.9
240	166,000,000	8.5	169,000,000	8.5	160,000,000	8.5	224,000,000	8.5	1,655,000,000	7.4
288									948,000,000	8.1
336										8.5
360									252,000,000	8.5

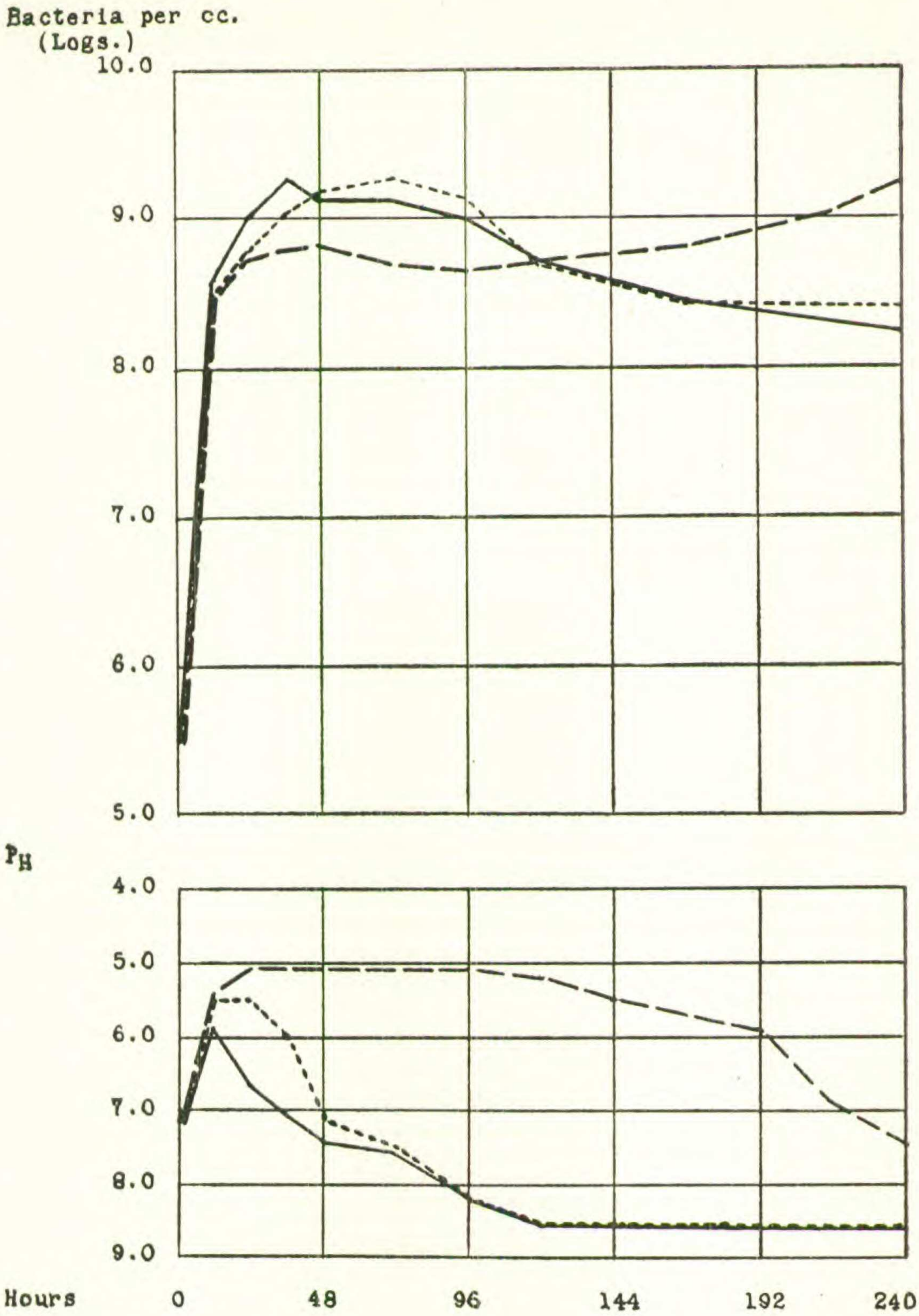

Fig. 7. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., variation in initial dextrose.

———— .1 per cent dextrose.
- - - - - - - - .2 per cent dextrose.
— — — — .3 per cent dextrose.

P_H 7.3, 7.1, 7.6, 7.5, and 7.4. The relation between growth curves and P_H curves, fig. 7, would indicate that *Bacillus coli* is more sensitive to alkali than to acid and that amounts of alkali or acid considerably less than the fatal dose become prominent factors in inhibiting growth.

Supplementing the preceding table, table VIII gives the results of growth of *Bacillus coli* in 1 per cent, 2.5 per cent, and 5 per cent dextrose media. A synthetic bouillon was used for these cultures consisting of .5 per cent asparagin, .5 per cent K_2HPO_4, and the dextrose as indicated. The growth and hydrogen ion concentration curves are plotted in fig. 8 on the same basis as the curves in all the other figures. As might be expected, the action in general corresponded to that of Culture 2, fig. 1, which was grown in 1 per cent dextrose. Both the growth and P_H curves showed a small lag at the beginning in 2.5 per cent dextrose and a greater one with some decrease in growth in 5 per cent dextrose. Following the initial lag, the cultures produced the usual growth, acid fermentation, and death. A slightly greater acid production occurred in the 5 per cent dextrose, for the hydrogen ion concentration went to P_H 4.7. The data of tables VII and VIII show that in cultures of *Bacillus coli* sufficient acid to kill the organisms was formed from 1 per cent or more of dextrose, while .15 to .3 per cent supplied only enough acid to inhibit the growth, and .1 per cent exerted a stimulative action. Thus the amount of dextrose present seems to regulate the reaction, which is a strong factor in growth and inhibition.

In connection with the reversion of reaction, the growth and inhibition of *Bacillus aerogenes* are of interest. One culture of plain bouillon and one culture of plain bouillon plus 1 per cent dextrose were inoculated with *Bacillus aerogenes;* the growth and hydrogen ion concentration changes are recorded in table IX and fig. 9. Both the growth and hydrogen ion concentration were very similar to those of *Bacillus coli* from the time of inoculation up to 96 hours. As fig. 9 illustrates, at 96 hours the abrupt descent of the growth curve was checked at 7,600,000 bacteria per cc. The slight drop to 5,000,000 bacteria per cc. in the next 48 hours was followed by a second rise which culminated in a maximum of 1,017,000,000 bacteria per cc. at 696 hours.

TABLE VIII

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., VARIATION IN INITIAL DEXTROSE IN ASPARAGIN BOUILLON

Cultures	1		2		3	
Dextrose	1.0%		2.5%		5.0%	
Hours	Bacteria per cc.	P_H	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	45,000	6.6	63,000	6.4	46,000	6.4
12	5,450,000	6.6	100,000	6.4	60,000	6.3
16	46,500,000	6.5	80,000	6.3	60,000	6.2
20	326,000,000	5.7	80,000	5.8	60,000	5.9
24	350,000,000	5.5	145,000	5.7	25,200	5.9
36	464,000,000	5.4	17,000,000	5.8	8,400	5.9
48	416,000,000	4.9	345,000,000	5.6	57,600	5.8
60	280,000,000	4.9	384,000,000	4.9	1,700,000	5.7
72	178,000,000	5.3	323,000,000	4.9	40,000,000	5.7
84	108,000,000	5.3	348,000,000	4.9	170,000,000	5.1
96	51,000,000	5.3	320,000,000	4.9	270,000,000	4.9
108	28,000,000	5.3	180,000,000	4.9	260,000,000	4.9
120	1,800,000	5.3	187,000,000	4.9	380,000,000	4.7
132	2,800,000	5.3	120,000,000	4.9	224,000,000	4.7
144	200,000		31,000,000		142,000,000	4.7
156	18,400	5.3	24,000,000	4.9	161,000,000	4.9
168	1,000	5.3	6,000,000	5.2	158,000,000	5.1
180	346	5.3	2,000,000	5.2	132,000,000	5.1
192	144	5.3	360,000	5.0	102,000,000	4.9
204	93		62,000		104,000,000	
216			391	5.2	54,000,000	5.3
240					21,000,000	5.3
264					9,900,000	5.0
300					900	5.0

Some points of interest in the P_H curves are that the high point, P_H 4.7, at 120 hours, did not occur during the period of greatest decrease in growth, that the P_H held at 4.9 for a considerable period after the second increase in growth began, and that the hydrogen ion concentration at the time when the maximum growth was reached was P_H 7.1. In plain bouillon there was no essential difference in growth or changes in hydrogen ion

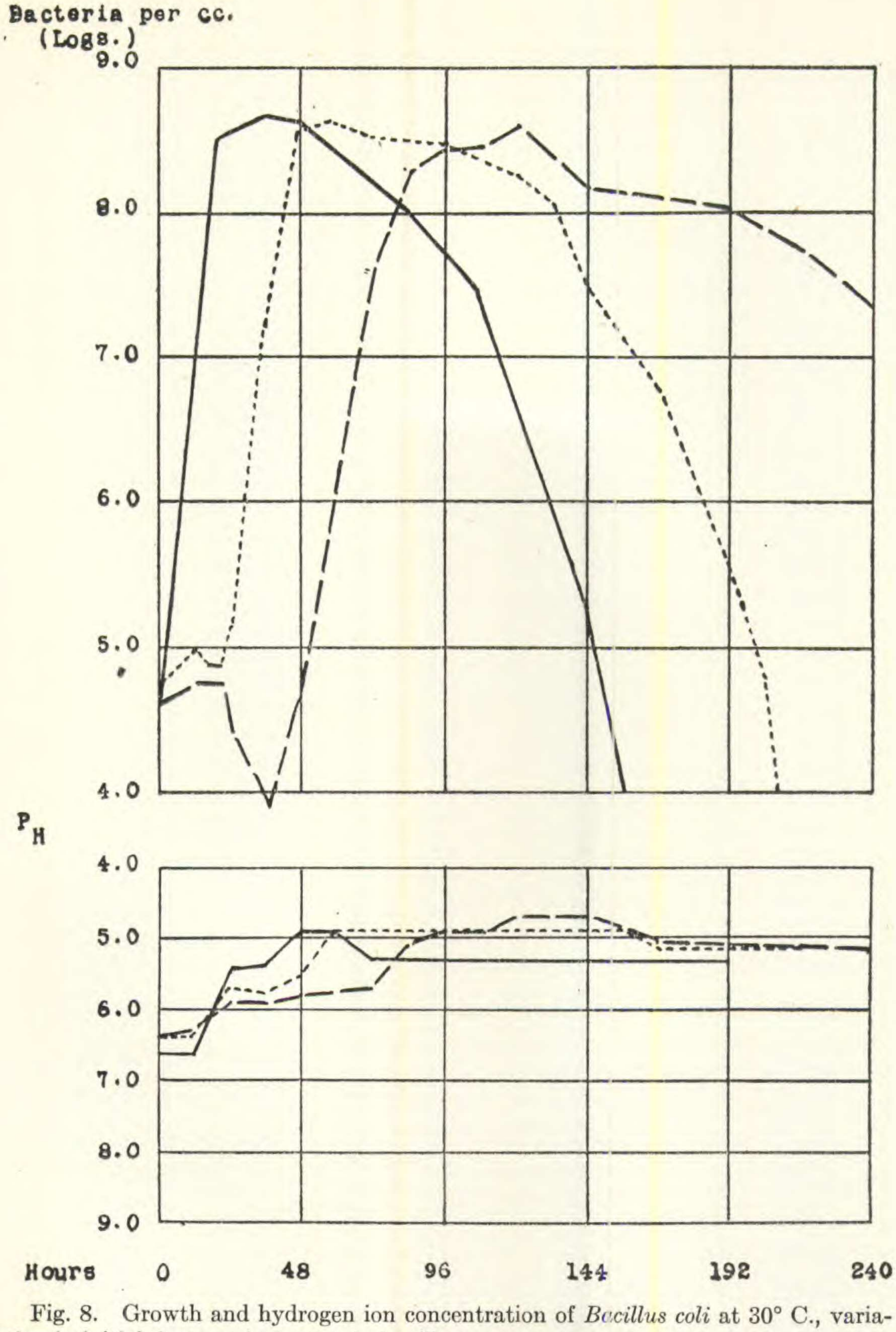

Fig. 8. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C., variation in initial dextrose in asparagin bouillon.

———— 1.0 per cent dextrose.
- - - - - - - - 2.5 per cent dextrose.
— — — — 5.0 per cent dextrose.

TABLE IX

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS AEROGENES AT 30° C., PLAIN AND 1 PER CENT DEXTROSE BOUILLON

Hours	1 Plain bouillon		2 Plain bouillon and 1% dextrose	
	Bacteria per cc.	P_H	Bacteria per cc.	P_H
0	98,000	6.9	94,000	6.9
12	171,000,000	6.9	272,000,000	5.5
24	236,000,000	7.0	300,000,000	4.9
48	306,000,000	7.4	139,000,000	4.8
72	455,000,000	7.8	27,000,000	4.8
96	307,000,000	8.1	7,600,000	4.8
120	169,000,000	8.3	6,300,000	4.7
144		8.3	5,000,000	4.9
168	162,000,000	8.3	10,000,000	4.9
192	121,000,000	8.3	23,000,000	4.9
216	108,000,000	8.3	38,000,000	4.9
264			125,000,000	4.9
312			211,000,000	4.9
360			539,000,000	5.8
408	37,000,000	8.3	815,000,000	6.6
456	44,000,000	8.5	836,000,000	6.9
504			761,000,000	6.9
552	36,000,000	8.6	775,000,000	6.9
624	20,000,000	8.7	927,000,000	6.9
696	22,000,000	8.7	1,017,000,000	7.1
864	8,640,000	8.7	690,000,000	7.3
1032	2,520,000	8.7	643,000,000	7.0
1200	7,830,000	8.7	485,000,000	7.6

concentration between *Bacillus coli* (table I, Culture 1) and *Bacillus aerogenes* (table IX, Culture 1). There is a similarity in growth of *Bacillus coli* in .3 per cent dextrose and *Bacillus aerogenes* in 1 per cent dextrose, the difference between the organisms apparently being in the greater resistance of *Bacillus aerogenes* to acid.

While the experimental data reported above have emphasized the importance of the H and OH ions as factors in inhibition, these ions do not represent the only products of metabolism which might be considered as inhibitory to growth. Ayers and

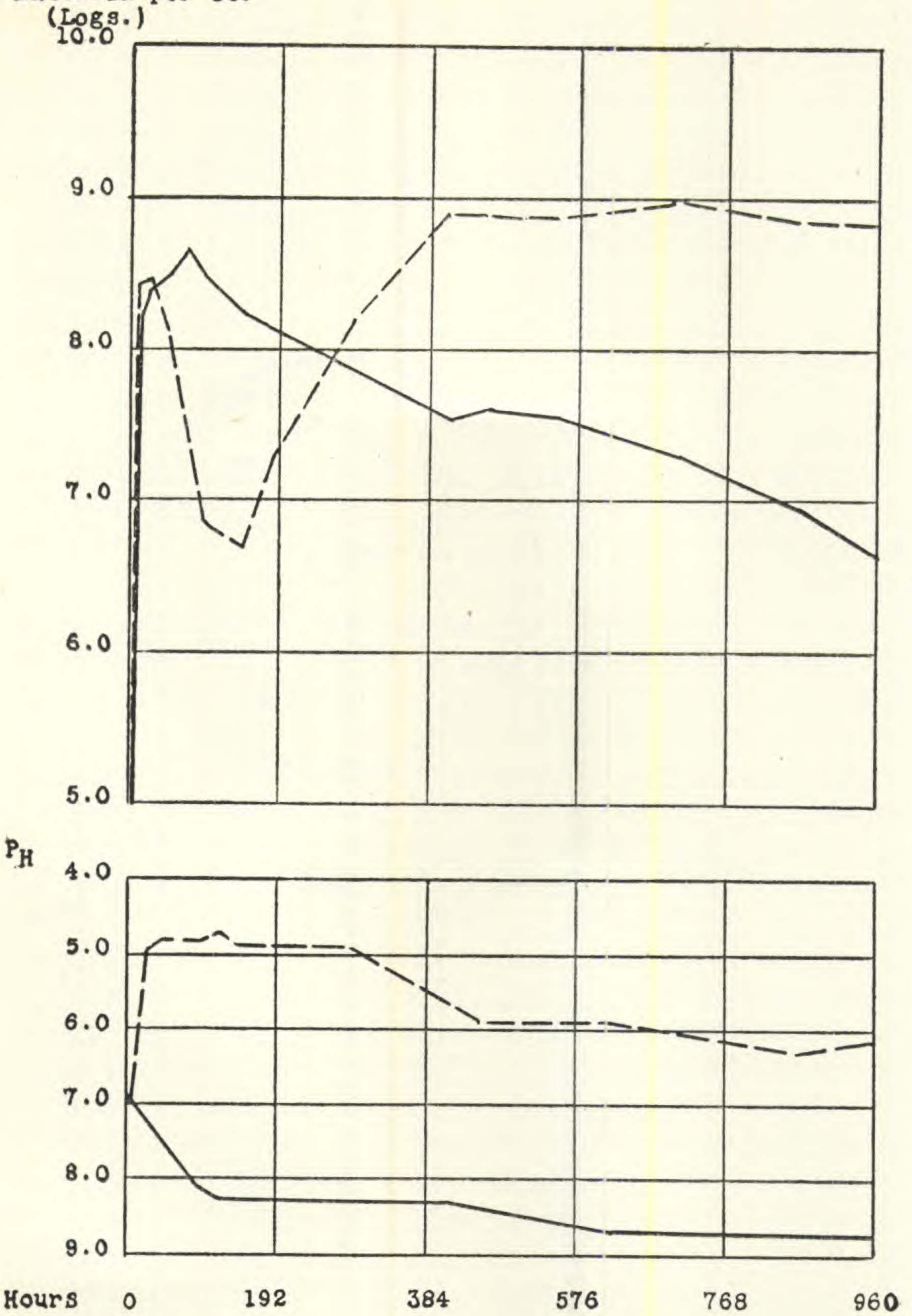

Fig. 9. Growth and hydrogen ion concentration of *Bacillus aerogenes* at 30° C.
———— plain bouillon.
— — — — 1 per cent dextrose bouillon.

Rupp have made quantitative determinations of formic, acetic, lactic, and succinic acids from *Bacillus coli* in a dextrose bouillon, and Wyeth, and Cohen and Clark have shown that the critical hydrogen ion concentration varies with the different acids, hydrochloric, acetic, and lactic, indicating that the anions of the acids or perhaps the undissociated molecules, as Winslow and Lochridge suggested, are also concerned in inhibition. Most of the work on the inhibitory effect of different acids has been based on inoculation of media of different P_H values obtained by using different acids, and the inhibition has been determined according to the presence or absence of growth after a certain interval. Such a method does not take into consideration milder phases of inhibition which are not severe enough to cause the death of the organisms. To illustrate this phase and to indicate some of the relations of the hydrogen ion factor to the other factors, the results of an experiment are presented in table x and fig. 10. Culture 1 was grown in 1 per cent dextrose bouillon, and Culture 2 in plain bouillon to which sterile N/5 HCl was added, as indicated in table x, in an attempt to simulate in plain bouillon the P_H curve of a culture fermenting dextrose bouillon, such as Culture 1. As seen from fig. 10, the culture produced alkali continually so that it was only possible by frequent additions of acid to hold the P_H in a zone around P_H 4.8, the greatest hydrogen ion concentration which Culture 1 attained. The growth curve, fig. 10, shows marked acid inhibition with almost no further increase in growth after the first addition of acid at 14 hours. There is practically no difference in the growth curves of the two cultures up to 72 hours, but from that point they separate widely, for death occurs shortly in the dextrose media and growth in Culture 2 does not go below 26,000,000 bacteria per cc. Thus a hydrogen ion concentration of P_H 4.8–4.9 when produced by the acid fermentation of dextrose was fatal, while that of P_H 4.7–5.1 from HCl was only strongly inhibitory, indicating that the other metabolic products of dextrose fermentation, such as acetate or lactate ions, evidently enter as factors in causing the death of the culture.

TABLE X

GROWTH AND HYDROGEN ION CONCENTRATION OF BACILLUS COLI AT 30° C., 1 PER CENT DEXTROSE BOUILLON AND PLAIN BOUILLON + HCl AT INTERVALS

Hours	1 1% dextrose bouillon		2 Plain bouillon + HCl		
	Bacteria per cc.	P_H	Bacteria per cc.	Cc. of N/5 HCl added	P_H
0	55,000	7.1	57,000		7.1
12	268,000,000	5.3	179,000,000		6.7
14				15	5.2
20	281,000,000	5.1			
24	214,000,000	4.9	178,000,000		5.3
26				4	5.0
36	220,000,000	4.8	197,000,000		5.2
38				5	4.9
48	189,000,000	4.8	175,000,000		5.1
50				5	4.7
60					4.9
72	119,000,000	4.8	116,000,000		5.0
74				3	4.7
96	14,500,000	4.9	77,000,000		4.9
120	11,100	4.9	96,000,000		5.3
122				3	4.9
144	0	4.9	55,000,000		5.1
146				2	4.9
168			26,000,000		4.9
192			60,000,000		5.1
194				2	4.9
234			74,000,000		4.9
276			144,000,000		5.3
278				4	4.9
348			148,000,000		5.1
350				3	4.8
492			95,000,000		4.9
612			73,000,000		5.1

Discussion

For a discussion of the combined results embodied in the experimental data presented, the inhibitory products of metabolism of *Bacillus coli* are divided under four topics: "autotoxins"; H ions; OH ions; and products of dextrose fermentation other than those directly related to changes in H ion concentration.

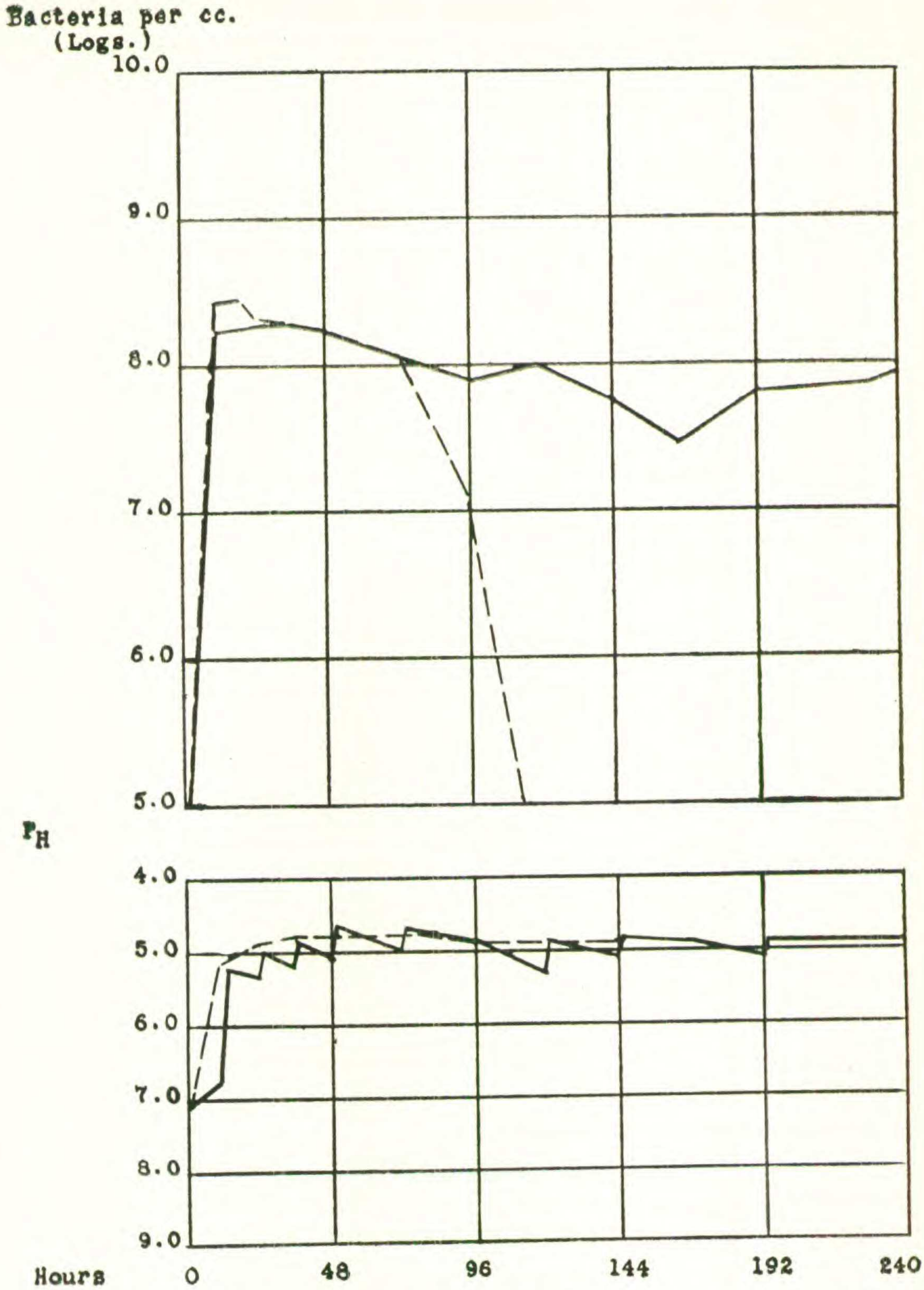

Fig. 10. Growth and hydrogen ion concentration of *Bacillus coli* at 30° C.
———— plain bouillon+HCl at intervals.
— — — — 1 per cent dextrose bouillon.

It appears improbable that *Bacillus coli*, grown under the conditions of the experiments reported, produces any "autotoxin" or special inhibitory substance such as Eijkmann and others claimed. The results given in tables II and III indicate that the slight inhibition increasing with the age of the culture is probably associated with a diminution in nutrients. No thermolabile product could be detected which would inhibit growth if the hydrogen ion concentration were corrected. It is difficult to reconcile the production of an enzymatic inhibiting substance with such growth as appears in figs. 1 and 6, especially as death did not occur in these cultures. In plain bouillon, fig. 1, the culture was viable after 75 days; and in dextrose bouillon, fig. 6, with the hydrogen ion factor controlled, growth attained 3,750,000,000 bacteria per cc. in 7 days, and there were still present over 2,000,000,000 bacteria per cc. after 840 hours. In addition there was no indication in the cultures in which death occurred that death could be attributed to an "autotoxin."

There is a direct relation between hydrogen ion concentration and inhibition. If the acid is formed from the fermentation of dextrose, with *Bacillus coli*, fig. 7 and table VII, there is no indication of acid inhibition at P_H 5.9 if maintained for only a short time. Some inhibition is apparent at P_H 5.5 which increases with the time that the culture is exposed to this P_H. There is a marked inhibition from an exposure of 72 hours to P_H 5.1, fig. 7, but it is insufficient to cause death. Fig. 4, however, shows that a prolonged hydrogen ion concentration of P_H 5.1 is lethal, and in every case throughout the experimental work a P_H of 4.9, when produced by acid fermentation of dextrose, proves fatal, figs. 1, 2, 3, 4, and 8.

To illustrate the relationship between hydrogen ion concentration and growth, four curves from figs. 1, 6, and 7 are assembled in fig. 11. The highest growth curve, No. 2, is attained in the culture in which the P_H remains practically neutral; the P_H of 5.1, No. 3, produces an intermediate growth; and the slight difference in hydrogen ion concentration between P_H 5.1, No. 3, and 4.9, No. 4, is fatal.

The OH ions also prove to be inhibitory according to the plain bouillon growth curve in fig. 11. An alkalinity corre-

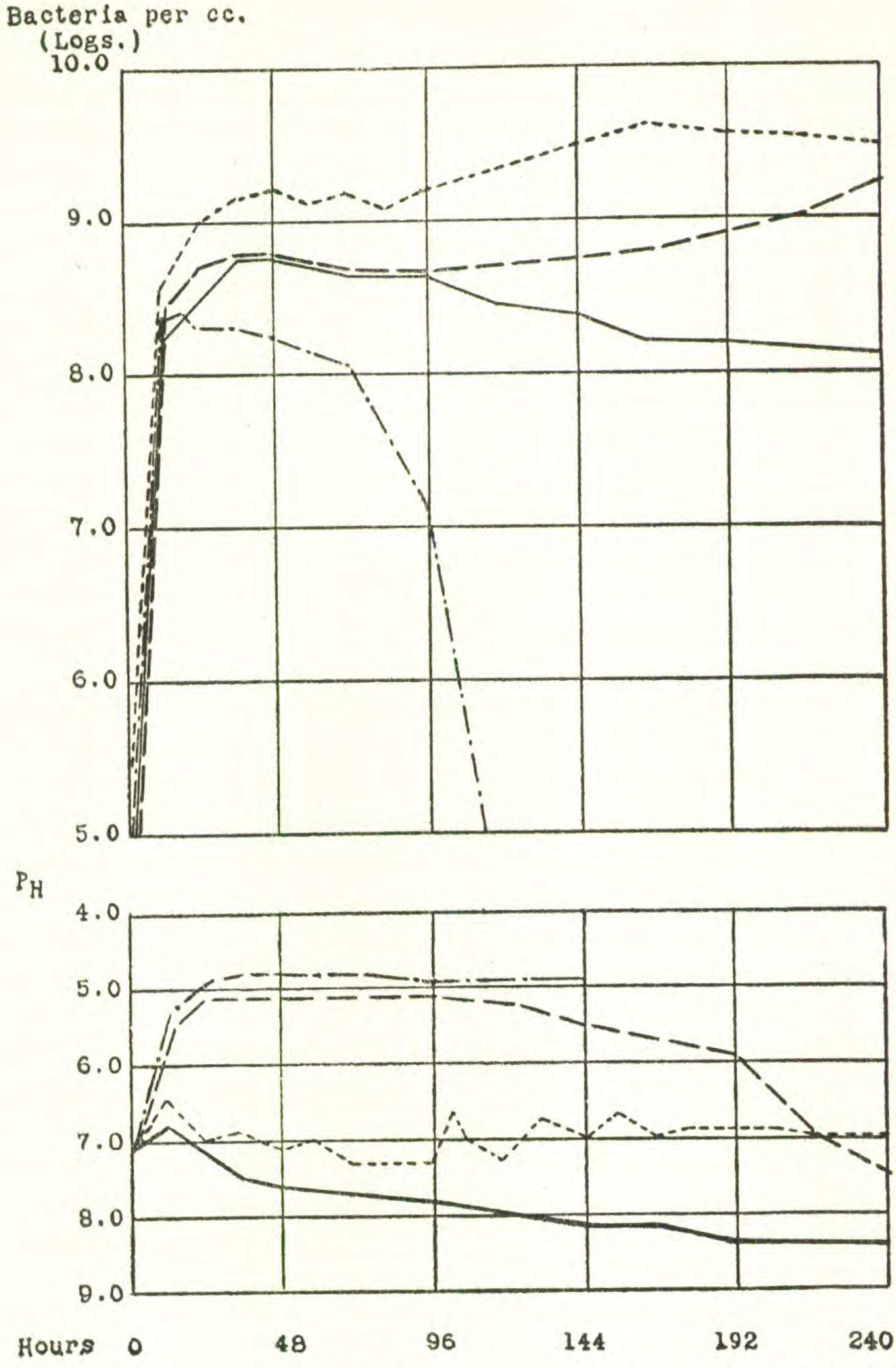

Fig. 11. Relation of growth to hydrogen ion concentration of *Bacillus coli* at 30° C.

No. 1 ———— plain bouillon.
No. 2 - - - - - - - - dextrose added at intervals.
No. 3 — — — — .3 per cent dextrose.
No. 4 —·—·—· 1 per cent dextrose.

sponding to P_H 7.6–7.8 is comparable in toxicity with an acidity of P_H 5.1. *Bacillus coli* seems more sensitive to small amounts of alkali than to small amounts of acid, for in the reversions of reaction in fig. 7, inhibition is evident shortly after crossing the neutral line, about P_H 7.1–7.6. In a freshly inoculated culture without dextrose, fig. 7, inhibition is first noted about P_H 7.5. While the hydroxyl ions appear more toxic to *Bacillus coli* in less concentration than the hydrogen ions, they do not seem to be fatal in greater concentration, for death of the culture was not observed on the alkaline side, although one culture containing $CaCO_3$, which is not reported in the data, carried the P_H to 9.5.

The importance of the factors other than H or OH ions which may enter into the inhibition or killing of a culture of *Bacillus coli* is not overlooked, but it should not be over-emphasized. For example, in a 1 per cent dextrose bouillon culture, such as is shown in fig. 1 or 10, in addition to the H ions, the anions, formate, acetate, lactate, and succinate, are formed (Ayers and Rupp, '18), probably other anions, and also the undissociated acids. These add their inhibitory action to that of the H ions in producing death at P_H 4.9, illustrated by fig. 10. The best growth curve, however, fig. 11, has only the hydrogen ion concentration controlled and in reality ferments much more dextrose than the 1 per cent dextrose culture of fig. 10. The former culture ferments a total of over 1.36 per cent and none of the products are removed from the culture, while the latter does not ferment all of the 1 per cent dextrose furnished. It seems possible that the metabolic products other than the H ions are not sufficiently inhibitory to influence greatly the growth until the hydrogen ion concentration approaches the acid limit, but toward the critical acid zone their effect becomes noticeable.

The growth curves as a whole do not agree exactly with the life phases presented by Buchanan ('18). In fact, the diversity of growths produced by varying the hydrogen ion concentration, as illustrated by fig. 11, is so great that one curve can express the growth of *Bacillus coli* in bouillon only when quite definite limitations of conditions are imposed. In a growing culture of an organism like *Bacillus coli* which produces acid from dextrose and alkali in plain bouillon, growth can be con-

trolled to a certain extent by the hydrogen ion concentration, which can in turn be controlled by the amount of dextrose furnished. The initial amount of dextrose determines the amount of acid produced or the maximum hydrogen ion concentration attained. The work of Clark and Lubs, Besson, Ranque and Senez, and the experimental data presented here give a rather definite idea of the action of *Bacillus coli* according to the amount of dextrose in the medium. With .3 per cent or less of dextrose, insufficient acid is produced to kill the organisms; .4 per cent or more is sufficient dextrose to produce acid to P_H 4.9 or better, and the culture becomes sterile in 6 days or less. An amount of dextrose not accurately determined, but between .3 and .4 per cent, probably depending to some extent on the buffer in the medium, should produce just enough acid, between P_H 5.1 and P_H 4.9, depending on the time of exposure, to kill the culture. If insufficient acid to kill the culture is produced, as from .3 per cent or less of dextrose, a reversion of reaction takes place, which Ayers and Rupp have explained with *Bacillus aerogenes* as the formation of alkaline carbonates from the organic acids, especially from the formic and acetic acids. There is a similarity in reaction and in growth curves between *Bacillus aerogenes* and *Bacillus coli*, the main difference appearing to be in the greater acid resistance of *Bacillus aerogenes*. Growth in the cultures where reversion of reaction takes place seems to be typical. One-tenth per cent of dextrose provides a stimulation to growth, but greater amounts produce some evidence of acid inhibition, followed by an increase in growth with the reversion of the reaction and alkaline inhibition between P_H 7.0 and 7.6. The least inhibition is found in a culture in which the hydrogen ion concentration is held in a narrow zone around the neutral point—probably P_H 6.0–7.0 is the best—by adding small amounts of dextrose at frequent intervals. Thus, with *Bacillus coli*, hydrogen ion concentration and growth within limits can be manipulated by the dextrose furnished. The growth curves emphasize not only the value of the initial reaction and composition of the medium, but also the importance in physiological studies of following the changes in hydrogen ion concentration which the growing bacteria produce in their substrates.

Summary

Growth and death of *Bacillus coli* in the culture bouillon of these experiments does not follow a constant curve but is dependent on the hydrogen ion concentration of the medium.

The hydrogen ion concentration of a growing culture of *Bacillus coli* is controlled by the composition of the medium, and particularly by the amount of fermentable carbohydrate present.

The maximum count, determined by the plate method, in the culture with the hydrogen ion concentration controlled is 3,750,000,000 bacteria per cc., as contrasted with a maximum of 281,000,000 bacteria per cc. in the 1 per cent dextrose bouillon with the hydrogen ion concentration uncontrolled.

No investigation was made of the limiting influence of other factors, such as aëration, on the maximum number of bacteria per cc. in the culture where the hydrogen ion concentration was controlled.

No metabolic product of the nature of an "autotoxin" could be found.

Of the products of metabolism, acid is the most inhibitory, checking growth slightly at P_H 5.5 and increasing in intensity to a lethal concentration between P_H 5.1 and 4.9.

The first inhibition on the alkaline side is noted between P_H 7.0 and 7.6, depending on the age of the culture and other factors. P_H 7.6 is comparable in inhibitory action to P_H 5.1. In an asparagin-$CaCO_3$ bouillon, P_H 9.5 is not fatal.

The inhibitory action of the metabolic products of dextrose other than the hydrogen ions is only evident near the critical acid concentration.

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